Nutrition, HIV, and Drug Abuse: The Molecular Basis of a Unique Role for Selenium

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This is a non-final version of an article published in final form in:

Taylor, E. W., Cox, A. G., Zhao, L., Ruzicka, J. A., Bhat, A. A., Zhang, W., Nadimpalli, R. G. & Dean, R. G. (2000). Nutrition, HIV, and Drug Abuse: The Molecular Basis of a Unique Role for Selenium. *JAIDS Journal of Acquired Immune Deficiency Syndromes*, 25, S53–S61.

Abstract:

HIV-infected injection drug users (IDUs) often suffer from serious nutritional deficiencies. This is a concern because plasma levels of micronutrients such as vitamin B_{12}, zinc, and selenium have been correlated with mortality risk in HIV-positive populations. Injection drug use also increases lipid peroxidation and other indicators of oxidative stress, which, combined with antioxidant deficiencies, can stimulate HIV-1 replication through activation of NF-κB transcription factors, while weakening immune defenses. As detailed herein, these prooxidant stimuli can also increase the pathogenic effects of HIV-1 by another mechanism, involving viral selenoproteins. Overlapping the envelope coding region, HIV-1 encodes a truncated glutathione peroxidase (GPx) gene (see #6 in reference list). Sequence analysis and molecular modeling show that this viral GPx (vGPx) module has highly significant structural similarity to known mammalian GPx, with conservation of the catalytic triad of selenocysteine (Sec), glutamine, and tryptophan. In addition to other functions, HIV-1 vGPx may serve as a negative regulator of proviral transcription, by acting as an NF-κB inhibitor (a known property of cellular GPx). Another potential selenoprotein coding function of HIV-1 is associated with the 3′ end of the *nef* gene, which terminates in a conserved UGA (potential Sec) codon in the context of a sequence (Cys-Sec) identical to the C-terminal redox center of thioredoxin reductase, another cellular regulator of NF-κB. Thus, in combination with known cellular mechanisms involving Se, viral selenoproteins may represent a unique mechanism by which HIV-1 monitors and exploits an essential micronutrient to optimize its replication relative to the host.

Keywords: Glutathione peroxidase | HIV | NF-κB | Selenium | Selenoprotein

Article:

Several studies have demonstrated that the intake or blood levels of various micronutrients are significantly correlated with survival in HIV-1 infection, both in HIV-infected injection drug users (IDUs), and in general HIV-positive populations (l-3). Of all micronutrients, the most widely documented and statistically significant correlation with HIV disease outcome has been

demonstrated for selenium (Se) status, which has been correlated with various indicators of disease progression, and is an independent predictor of mortality in HIV infection (3-5), as reviewed previously (6). This is of particular interest, because Se, although classified as a mineral, is a potent dietary antioxidant, and its cellular actions are intimately linked to the redox status of the cell, and to the redox regulation of genes that are important for various immune cell functions (7). Thus, given an accumulating body of evidence that oxidative stress is a hallmark of AIDS (8,9), and an activator of HIV-1 replication in vitro (10,11), it is not surprising that Se should prove to be of critical importance in HIV-1 infection.

SELENIUM AND CELLULAR IMMUNITY

In addition to nonspecific incorporation as selenomethionine, Se can be specifically incorporated into proteins as the rare amino acid selenocysteine (Sec), the Se analogue of cysteine. In mammals, Se has many critical functions, including antioxidant defense, thyroid action, reproductive function, and immune function (12). These biologic functions of Se are generally associated with specific selenoproteins; for instance, Se is critical for antioxidant defenses largely because it is an essential component of Se-dependent glutathione peroxidases (GPx).

It is now well established that adequate levels of Se are necessary for the immune system, and cellular immunity in particular, to function properly (7,13). Se supplementation increases the cytotoxicity of killer cells as well as their proliferation in response to mitogens and antigens (14), whereas Se deficiency has the opposite effect, and is commonly associated with impaired immune function (7). Correlations between CD4+ T-cell counts and plasma Se levels have been widely documented, most significantly in HIV-infected patients (6). Se also potentiates the action of the cytokine interleukin 2 (IL-2), by upregulating the IL-2 receptor (15).

Significantly, the mRNA for a selenoprotein gene called Sps2, involved in biosynthesis of selenophosphate, the activated Se species required for formation of selenocysteine, is upregulated on activation of T lymphocytes (16); this suggests that selenoprotein synthesis is required for some aspect of T-cell function. Because cellular immunity is our primary defense against both cancer and viruses, a role of Se in cellular immune function represents a common factor in the known anticancer (17) and antiviral benefits of dietary Se (18).

DRUG ABUSE, MALNUTRITION AND IMMUNE FUNCTION

Injection drug users are a population known to be exceptionally prone to malnutrition, for several reasons. An addict's prime concern is obtaining the next drug dose; obtaining food is secondary. Furthermore, most IDUs are socially marginalized and impoverished, in part due to the cost of supporting their drug habit, so that the quality of their diet is often inadequate. These nutritional deficiencies associated with drug addiction have been linked to compromised immune function even in HIV-negative drug users (19), which is not surprising in light of the importance of nutrition for maintaining optimal immune function (20).

Of particular significance for the role of antioxidants in HIV-positive IDUs is that *injection drug use exerts an exceptional oxidant stress on the body and the immune system.* In part because of tissue damage and cellular injury, drug abusers show increased lipid peroxidation products in

blood, liver, and urine (21-23), and low serum thiol levels indicating increased oxidative stress are predictive of outcome in HIV-positive IDUs (24). This effect of drug abuse leads to an increased requirement for nutritional antioxidants, which is unlikely to be met in typical malnourished drug users. A study of heroin users showed significantly low urinary Se levels (25), which suggests that dietary Se intake is low or that absorption is impaired in IDUs. This would exacerbate the oxidative stress that is a direct consequence of injection drug use.

The now well-established correlation between Se status and HIV-1 disease progression (3-6) should be particularly apparent in HIV-positive IDUs, in the light of the facts just reviewed. To summarize:

Se is essential for cellular immunity, as well as for antioxidant defenses.

Malnutrition is common in drug addicts.

Injection drug use can be associated with increased oxidative stress and lipid peroxidation.

HIV-I transcription is activated by oxidative stress, through the transcription factor nuclear factor KB (NF-κB).

In essence, the multiple risk factors of increased oxidative stress and inadequate (dietary) antioxidant intake can activate HIV-1 replication through known mechanisms, while weakening immune defenses. The logic of this argument is supported by the finding that the most significant correlation to date between Se status and HIV-1 disease outcome was observed in a cohort of HIV-positive IDUs, in whom low plasma Se was associated with a 20-fold increased risk of AIDS-related mortality (3).

THE CELLULAR SELENOPROTEINS GPx AND THIOREDOXIN REDUCTASE REGULATE TRANSCRIPTION FACTOR NF-κB

In the cell nucleus, by binding to a specific DNA consensus sequence, the active form of NF-κB acts as a transcriptional activator of a number of immune-related genes, including various cytokines, adhesion molecules, and genes that regulate cell proliferation and apoptosis (26-28). By incorporating DNA sequences to which NF-κB can bind into its promoter region, HIV has evolved so that it can be activated by the same cellular factor that stimulates these various immune-related functions.

The activity of NF-κB is regulated in a complex manner by cellular location and redox status (26). Initial activation in the cytosol occurs through separation of the inhibitory KB subunit I-κB, which exposes a nuclear translocation signal on NF-κB, so that active NF-κB dimers can enter the nucleus and bind to their cognate DNA sites. This cytosolic activation is stimulated by oxidative stress (e.g., peroxides), inflammatory cytokines (e.g., tumor necrosis factor-a) and various noxious stimuli including ultraviolet light and radiation, in all of which the formation of reactive oxygen species is a common factor. Thus, oxidative stress is a well-documented activator of NF-κB (26-28), and of HIV transcription through NF-κB activation (8-11).

It is therefore not surprising that in cell culture studies Se, as a component of the selenoprotein GPx, has been consistently found to be an inhibitor of NF-κB activation, presumably due to the reduction of peroxide levels by GPx (11,28,29). However, although those and many other cell culture studies have demonstrated that, in general, oxidants stimulate whereas antioxidants inhibit NF-κB activation and HIV-1 transcription, activation of NF-κB does not involve exclusively oxidative stimuli. Despite the important role of oxidative stress in the cytosolic activation of NF-κB, to bind DNA in the nucleus, the thiol group of an active site cysteine residue of NF-κB must be in a reduced state (26). Thus, although oxidative stress appears to be a primary activator of NF-κB, this must be balanced by the action of selective cellular redox systems that, in the midst of this oxidative environment, can provide reducing power to maintain NF-κB in its active, reduced state. This reductive activation of NF-κB is one major functions of the selenoprotein thioredoxin reductase (TrxR) and its substrate thioredoxin (26,30).

GPx and TrxR are two of the most widely distributed selenoproteins, and it is remarkable that both have been demonstrated to be potent regulators of NF-κB activity, and all the more so that they appear to have opposed functions, that is, GPx inhibits (11,28,29) whereas TrxR activates (26,30) NF-κB activity. It is difficult to say which of these two may predominate, because the well-documented inhibition of NF-κB by selenite in cell culture may be due in part to the direct formation of inactive Se adducts at relatively high concentrations (>5 μM), and thus, rather than being mediated entirely through selenoproteins, a portion of this inhibition may represent a unique effect of selenite ion (27). Nonetheless, compelling evidence indicates that, under normal physiologic conditions, the selenoproteins GPx and TrxR are major regulators of NF-κB activity. This is of considerable significance in the light of evidence presented here subsequently that HIV-1 encodes selenoprotein modules having distinct similarities to both GPx and TrxR.

AN HIV-I-ENCODED SELENIUM-DEPENDENT GPx GENE

An *env* gene variant associated with one of the potential -1 frameshift sites predicted in HIV-1 in 1994 (31) was subsequently identified as a virally encoded homologue of GPx, the prototypical mammalian selenoprotein (6). This viral GPx (vGPx) module is encoded overlapping the HIV-1 *env* gp41 coding region in the -1 reading frame; thus the gene was tentatively named *env-fs,* for *env* frameshift. It contains a single UGA codon (potentially encoding Sec) near the middle of a small but highly conserved novel open reading frame, with a conserved -1 frameshift signal near its 5' end. The UGA codon is well conserved in the predominant American and European subtype B, but is found to be an AGA (Arg) codon in most other HIV-1 subtypes, a substitution that may still be compatible with GPx activity (6). The translated HIV-1 *env-fs* sequence contains a common variant of the GPx active site consensus sequence spanning the catalytic Sec *(U* in Fig. 1).

Using a sensitive multiple sequence comparison method, a strong similarity between the entire HIV-1-encoded *env-fs* sequence and the aligned set of GPx sequences was demonstrated. As previously aligned (6), the similarity score of this novel HIV sequence versus an aligned group of GPx sequences is five standard deviations (SD) above the average similarity score of randomized sequences of identical composition. That significance score increases to-6.3 SD based on the revised alignment shown in Figure 1.

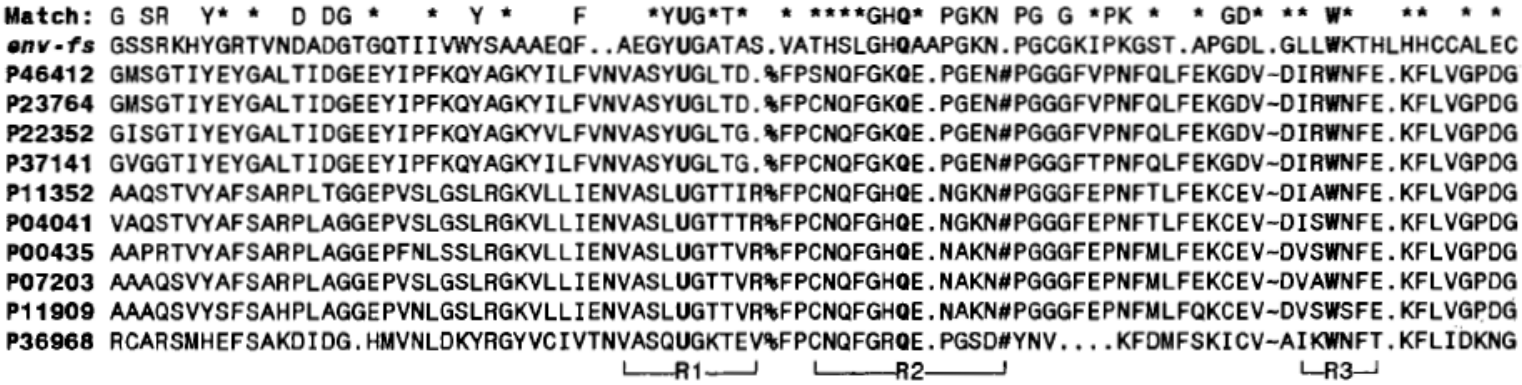
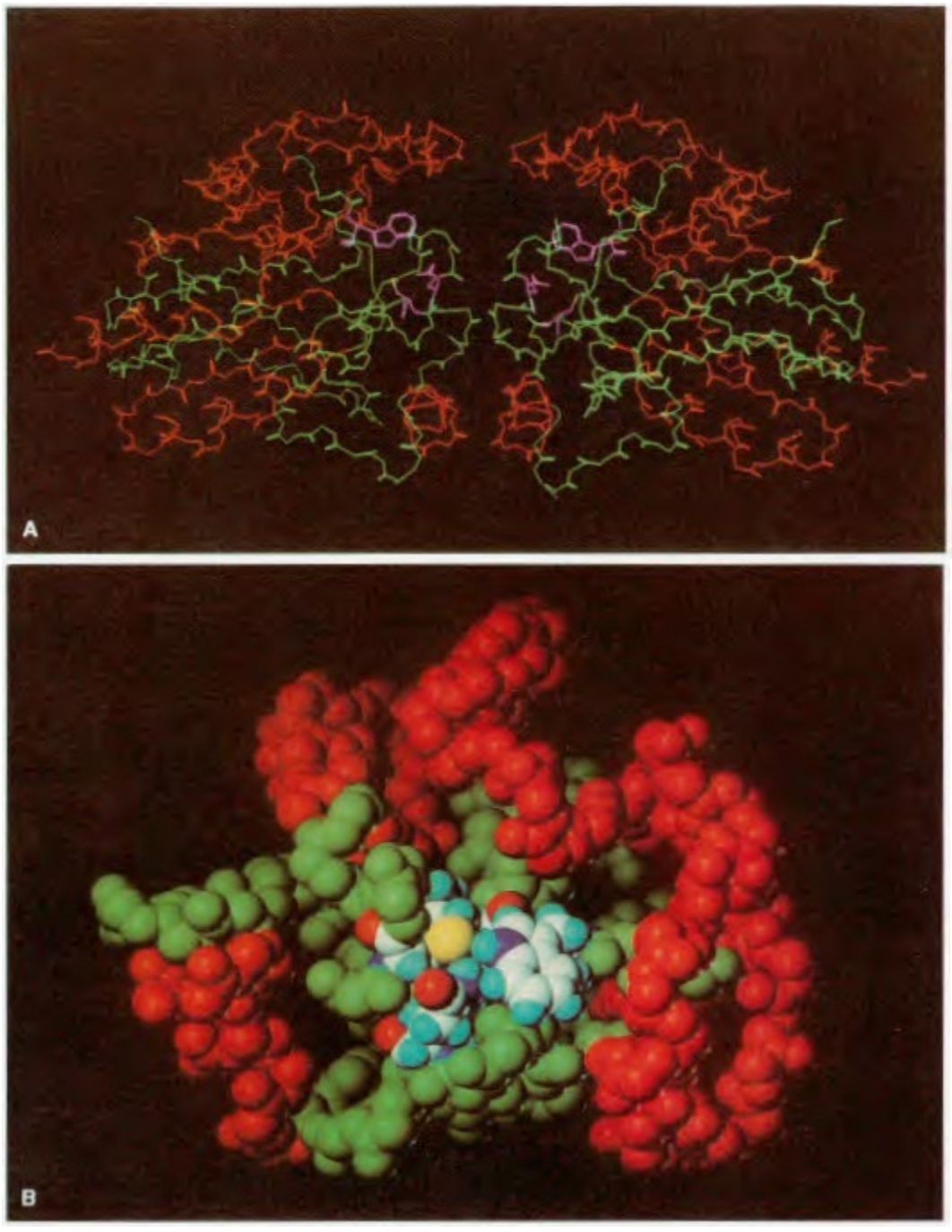


FIG. 1. Sequence alignment of HIV-1 *env-fs* sequence versus Se-dependent glutathione peroxidases. Sequences are single letter amino acid codes, with U as the symbol for selenocysteine, Sec, encoded by the UGA codon in RNA. GPx sequences are listed by Swiss-Prot database accession number. *Env-fs* amino acids identical to one or more of the aligned GPx sequences are shown as letters in the "match" line above the alignment; similar residues are indicated by an asterisk. Three active site regions that are adjacent in three-dimensional space are shown below the alignment, labeled R1 to R3. All three regions and their essential catalytic amino acids, Sec, Gin and Trp (U, Q and W respectively, shown highlighted in **bold**), are represented in the truncated *env-fs* sequence. The alignment is similar to that shown as Figure 1 of Taylor et al. (6) up to the end of R2, but is different beyond R2. There are three large deletions in *env-fs* relative to the GPx sequences, at the locations indicated by the symbols %, #, and ~ in the alignment. As computed using the alignment shown, that is, omitting the three internal GPx regions where there are major deletions in the truncated HIV-1 GPx homologue, the total similarity score of the *env-fs* sequence to the aligned GPx sequences is 6.3 standard deviations above the average similarity score computed for 100 optimally aligned randomly shuffled sequences of identical composition (see Experimental section).

We examined alternative alignments of the C-terminal region of *env-fs* to GPx in the light of the bovine GPx crystal structure and its active site (Fig. 2), and identified the viral sequence matching a third GPx active site region with a conserved Trp (W), shown as region 3 (R3) in the alignment of Figure 1. This reveals a total of three significant internal deletions in the HIV-1 sequence relative to the mammalian GPx sequences. These deletions in *env-fs,* involving 19, 11, and 41 residues, respectively, lie between the three conserved active site regions (YUG, GHQ, and W) and project away from the active site in three-dimensional (3-D) space (Fig. 2A, 2B).

To assess this proposed homology in structural terms, a 3-D molecular model of the HIV-1 vGPx (Fig. 2) was made by homology modeling, based on the alignment of Figure 1 (32). This showed that all three deletions are structurally feasible, although the first and third deletions would involve loss of domains that participate in formation of the tetrameric GPx enzyme. This suggests that the HIV-1 GPx homologue probably does not function as a multimer, which would make it similar to the phospholipid hydroperoxide GPx, which acts as a monomer (12). The proposed homology is also strongly supported by the fact that the Trp (W) active site residue in R3 (Fig. 1) is highly conserved in HIV-1 sequences. This conservation also provides compelling theoretical evidence that this is a functional gene, because the UGO Trp codon in the -1 frame is in the context of the sequence UCUGGA, which encodes Ser-Gly in the HIV envelope protein. Due to the degeneracy of the genetic code, Ser could be encoded using any base in the third codon position (i.e., UCN), so the conservation of a U in this position in HIV-1 sequences can only be explained by a functional requirement, such as the need for the UGO in the -1 frame (there is no known RNA structural element spanning this UGO codon). This putative HIV-1 vGPx gene has now been cloned and, when expressed as a selenoprotein in transfected

mammalian cells, was found to yield as much as a 99% increase in GPx activity relative to controls (32). Furthermore, the Cys homologue (Sec to Cys mutant) of the vGPx has been expressed in bacteria and isolated; the purified 9-kd protein has significant GPx activity, although that activity is low, as would be expected for a Cys mutant of a Se-dependent GPx (unpublished data).



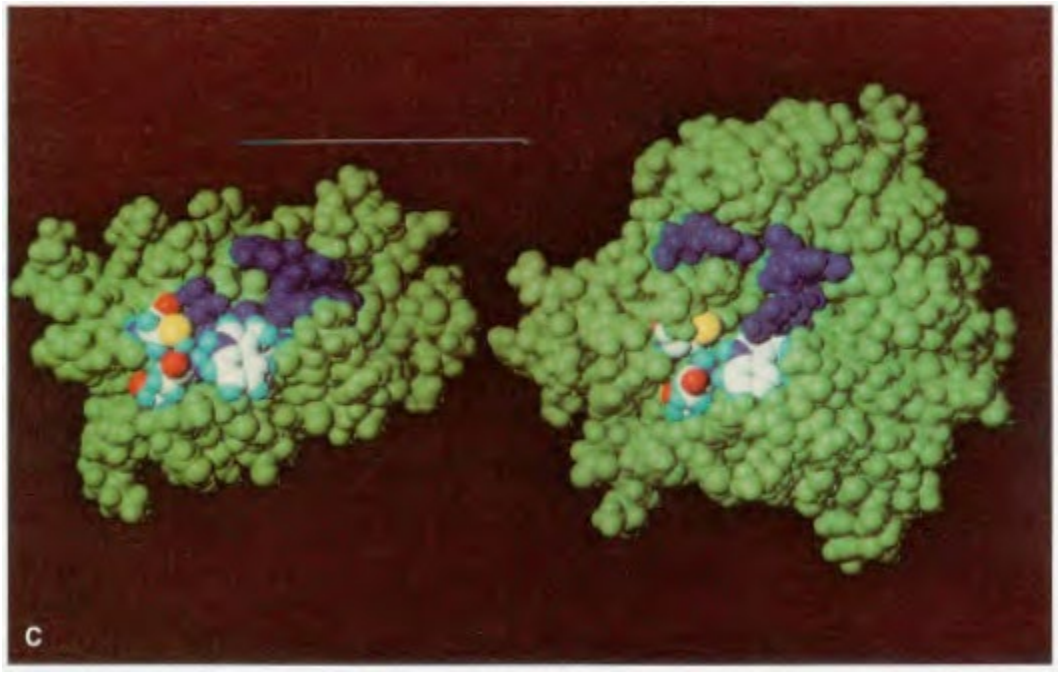


FIG. 2. Molecular modeling studies of the putative HIV-1 glutathione peroxidase (GPx) module. **(A)** View of the protein backbone of the x-ray crystal structure of the bovine GPx dimer (1GP1.pdb) with C-terminal and internal regions that are deleted from the HIV-1 GPx homologue shown in red, and the regions retained in HIV-1 *(env-fs* in the alignment of Fig. 1) shown in green, with three conserved active site residues (Sec, Gin and Trp) highlighted in magenta, with side chains displayed. The deleted regions (red) include a helix involved in dimer formation *(bottom, center)* and another region *(top)* that is involved in dimer and tetramer formation (tetramer not shown). The catalytic core of the enzyme is conserved in the truncated HIV-1 homolog. **(B)** A spacefill view of the protein backbone of the GPx monomer, colored as in A, except that the catalytic triad of Sec, Gin and Trp are shown in CPK colors. The deleted regions (red) all lie outside of the catalytic core domain. **(C)** *Left:* An all-atom homology model of the putative HIV-1 GPx with the deleted (red) regions shown in B removed and the resulting gaps reconnected by loop remodeling, giving a continuous peptide backbone matching the HIV-1 sequence *(env-fs* in Fig. 1). The active site triad is shown in CPK colors, with the Se atom in yellow. A cluster of basic residues (one Lys and several His) is shown in blue. *Right:* A similarly oriented and rendered all-atom view of the bovine GPx structure. The basic residues (blue) are all Arg, at least one of which is involved in binding glutathione. The HIV-1 GPx homologue, although highly truncated, includes the complete catalytic core of the enzyme, but it is likely to function as a monomer due to the deletion of regions involved in tetramer formation in the cellular GPx enzyme. The alignment of Figure 1 is structurally feasible, because the locations of the internal deletions permit the rest of the core structure to be maintained (32).

THE 3' TERMINAL OF HIV-1 *nef* IS A UGA CODON IN A MOTIF IDENTICAL TO THE TrxR C-TERMINAL REDOX CENTER

As detailed previously (18), the 3'-terminal UGA codon of *nef* is highly conserved in the known group M HIV-1 isolates (in >99% of known sequences), which is a unique feature of HIV -1 compared with HIV -2 and SIV *nef* genes, in which UAA and UAG stop codons are also found at the 3' end of *nef* in various strains and subtypes, with no particular bias. Conservation of this 3' -terminal UGA codon is perhaps the most striking difference between the *nef* gene of the more pathogenic HIV-] versus that of the other primate retroviruses. A selenoprotein coding function is a simple and appealing hypothesis, particularly because HIV- J encodes a vGPx gene upstream in the *env* region (6,32). Preliminary evidence suggests that readthrough suppression of the *nef* 3' UGA codon can occur in vivo, and that in vitro this effect is Se dependent (33). Furthermore,

additional results consistent with ^{75}Se incorporation in a *nef* isoform during in vitro translation of a patient-derived *nef* isolate have been obtained (Benjamin Blumberg, personal communication).

Because *nef* is highly expressed as an early gene, and no frameshift is required to encounter this in-frame 3'-terminal UGA codon during *nef* protein synthesis, we previously suggested that readthrough of this UGA codon, leading to sequestration of Sec in a *nef* selenoprotein isoform, might contribute to *nef*-associated pathogenicity of HIV-1 (18,31). However, until recently, there was little basis for developing a hypothesis as to the possible function of this *nef* isoform, other than a potential pathogenic role in intracellular Se depletion by the above mechanism.

The discovery that TrxR is a selenoprotein presents an intriguing possibility. Another feature of the *nef* 3' terminal is a conserved Cys residue immediately preceding the UGA codon. Thus, this HIV-1 encoded sequence is identical to that of the redox center of TrxR, where a geminal Cys-Sec pair al the protein C-terminal has been shown to be essential for the redox activity of TrxR (30). In addition, a sequence downstream of the UGA codon in *nef*, RRGLGGT(X_7)CCI, is quite similar to the sequence of a second disulfide redox center located near the N-terminal (#52-65) of TrxR, RWGLGGTC(X_4)CI.

It must be remembered that *nef*, along with *tat* and *rev*, is ex pressed early in the cell infection cycle, at a point when HIV needs 10 activate its transcription. If this hypothetical selenoprotein isoform or *nef* can, like TrxR itself, contribute to activation of NF-κB, while reducing cellular GPx levels through Sc sequestration (thereby producing oxidative activation of NF-K B), that would be perfectly consistent with the viral agenda at that point in the infection cycle, as exemplified by the transactivating effect of the HIV *tat* gene that is expressed simultaneously with *nef*. Thus, in the case of *nef*, we predict that HIV-1 is using a viral selenoprotein for NF-κB activation, whereas the HIV-1 vGPx—a late gene—would be expected to deactivate NF-κB by decreasing oxidant tone. Notably, another role of TrxR is in the synthesis or deoxyribonucleotides, through ribonucleolide reductase (30). DNA synthesis is certainly another function that could help explain why HIV-1 might encode a module with TrxR-like activity. This would be consistent with the enhancement or infectivity and stimulation or proviral DNA synthesis activities that have been demonstrated for the HIV-1 *nef* gene (34).

AN HIV-1-ENCODED NF-κB HOMOLOGUE?

An earlier study (31) noted the existence of a potential HIV-1 selenoprotein module potentially expressed by a -1 frameshift from the protease coding region, which was thus later named *pro-fs* (18). This was predicted to be a very basic, positively charged protein (pI = 11), suggesting a nucleic acid binding protein, database searches produced matches to various DNA binding proteins. In particular, Taylor el al. suggested a similarity to the papillomavirus E2 DNA binding protein, which has a conserved Cys residue that aligns with the highly conserved UGA codon of *pro-fs* (31).

This hypothesis can be refined in light of data contained in a paper that was in press (26) at the time our 1994 paper (31) was submitted. Significantly, like the E2 proteins, members of the NF-κB family of transcription factors also contain an essential Cys residue in their DNA-binding domain; this Cys is in fact the amino acid that TrxR maintains in a reduced (thiol) state in order

for NF-κB to bind to DNA (26,30). From a comparison of the alignment of *pro-fs* versus the E2 proteins in Figure 9 of Taylor et al. (31) with the alignment versus the DNA-binding "NRD" domain of the NF-κB family (Fig. 3), it is evident that the alignment or *pro-fs* to the NRD domain gives a tighter alignment, with no gaps in the primary DNA con tact region. The statistical significance of this similarity relative to randomly shuffled sequences is 4.5 SD for the region shown in Figure 3.

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Match:      IKI  Q KG  IR*R *C    *  *     T KN*R
pro-fs    VTIKIGGQLKGSSIRYRSRcYSIRRNEFARKMET.KNDR
                                   /
DIF-D     PHLRIVEEPTSNIIRFRYKCEGRTAGSIPGMNSSSETGK
DORS-D    PYVKITEQPAGKALRFRYECEGRSAGSIPGVNSTPEN.K
KBF1-C    PYLQIIEQPKQRGFRFRYVCEGPSHGGLPGASSE.KNKK
KBF1-H    PYLQILEQPKQRGFRFRYVCEGPSHGGLPGASSE.KNKK
KBF1-M    PYLQILEQPKQRGFRFRYVCEGPSHGGLPGASSE.KNKK
KBF2-C    PYLVIIEQPKQRGFRFRYVCEGPSHGGLPGASSE.KGHK
KBF2-H    PYLVIVEQPKQRGFRFRYGCEGPSHGGLPGASSE.KGRK
KBF3-H    PYLVIVEQPKQRGFRFRYGCEGPSHGGLPGASSE.KGRK
RELB-H    PHLVITEQPKQRGMPFRYECEGRSAGSILGESST.EASK
RELB-M    PYLVITEQPKQRGMRFRYECEGRSAGSILGESST.EASK
RELB-X    PELNITEQPKQRGMRFRYQCEGRSTGSILGEKST.EHNK
REL-A     PYIEIFEQPRQRGTRFRYKCEGRSAGSIPGEHST.DNNK
REL-C     PYIEIFEQPRQRGMRFRYKCEGRSAGSIPGEHST.DNNK
REL-H     PYIEIIEQPRQRGMRFRYKCEGRSAGSIPGEHST.DNNR
REL-M     PYVEIIEQPRQRGMRFRYKCEGRSAGSIPGERST.DNNR
TF65-C    PFVEILEQPKQRGMRFRYKCEGRSAGSIPGEHST.DSAR
TF65-H    PYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERST.DTTK
TF65-M    PYVEIIEQPKQRGMRFRYKCEGRSAGSIPGERST.DTTK
TF65-X    PPVEIIEQPKQRGMRFRYKCEGRSAGSIPGERST.DTSK
DNA recognition:      ************
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FIG. 3. The HIV-1 *pro-fs* sequence (31) compared with a multiple alignment of the DNA binding domain of the NF-κB/Rel/Dorsal (NRD) family of transcription factors; the region involved in sequence-specific DNA recognition is indicated. The following amino acid similarities are highlighted in **bold.** Basic: R, K, H (Arg, Lys, His); Aromatic: Y, F (Tyr, Phe); Hydrophobic: L, I, V, F (Leu, lie, Val, Phe); Acid/amide: D, E, N, Q (Asp, Glu, Asn, Gin). *Pro-fs* amino acids identical to one or more of the aligned NRD sequences are shown as letters in the "match" line above the alignment; similar residues are indicated by *. The underlined C in *pro-fs,* aligned with the conserved C of the NRD domain, is a potential Sec residue, encoded by a highly conserved UGA codon in HIV-1 . Statistical significance of the sequence similarity is 4.5 standard deviations relative to that expected for random sequences of identical composition. The region shown above corresponds to positions 8-46 of the alignment for Domain #1239 at the ProDom website (www.toulouse.inra.fr/prodom/doc/prodom.html), from which the full names of the NRD sequences shown can be obtained .

The protein sequence encoded in this overlapping gene region of protease is highly conserved in HIV-1 sequences. which cannot be explained by any known coding or functional requirement of HIV. Furthermore, using a standard assay, we have established that the predicted -1 frameshift site in the HIV-1 protease gene (31) is active in vitro (Fig. 4). This frameshift occurs just upstream of the *pro-fs* sequence (RYRSRC) that matches the "signature" of the NRD domain (RFRYXC). Given the combination of an active frameshift into this novel genomic region of

HlV-1, and a highly conserved sequence in the overlapping -l frame, there is little reason to doubt that this sequence is expressed in vivo.

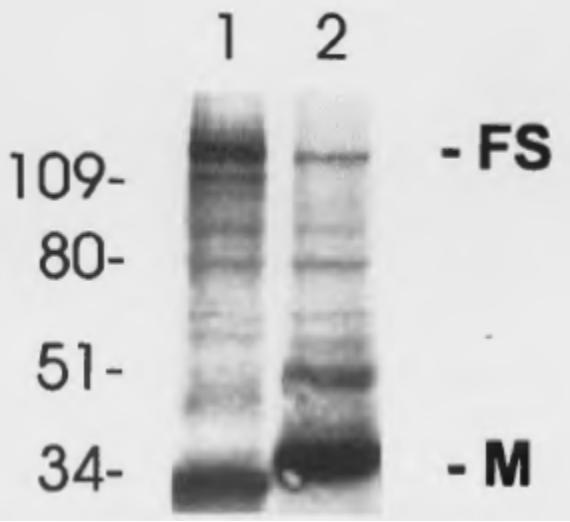

FIG. 4. In vitro translation (IVT) assay showing -1 frameshift activity induced by a fragment of the HIV-1 protease gene (31) when inserted into the RW-201 vector. In addition to a major low molecular weight "zero frame" translation product (about 40 kd, shown as M), if the insert can induce -1 ribosomal frameshifting, higher molecular weight minor products (full length product at about 150 kd, shown as FS) are observed. Lane 1: IVT of RW-201 with an insert from a high efficiency (20%) -1 frameshift sequence from the mouse mammary tumor virus, used as a positive control. Lane 2: IVT of RW-201 with a *pro-fs* insert, with the UGA stop codon in the -1 frame of the insert mutated to a sense codon to permit readthrough in the -1 frame. Note the FS band similar to lane 1 but less intense. No bands were visible in control lanes with no DNA added (not shown). See Experimental Section for details.

From the gene alignment, it is clear that the protease-processed form of *pro-fs* could be at the most a minimal DNA-binding domain, and lack the transcriptional activating (TA) domain of NF-κB. By analogy to the E2 proteins (31), a DNA-binding domain alone could function as a repressor by preventing the complete protein (DNA-binding plus TA domain) from binding, or by forming inactive heterodimers. Thus, *pro-fs* may function as a transcriptional silencer or repressor, which could serve to maintain latency in an infected cell, that is, keep the provirus from being overexpressed, perhaps to evade immune surveillance. In essence, processed *pro-fs* could function as an antagonist to NF-κB, which would be consistent with the clinical data on Se status and HIV-1 disease progression and outcome (3-6).

CONCLUSIONS

Not only does HIV-1 appear to encode homologues or catalytic motifs of selenoproteins known to regulate NF-κB, that is, its own GPx and a module in *nef* that is identical to the TrxR "redox center," but it actually may encode its own highly truncated NF-κB homologue *(pro-fs),* that has Se instead of S in its DNA-binding domain.

Although the precise mechanisms by which HIV-1 may recode specific UGA codons as sense codons for Sec have not yet been established, the sequence similarities to known selenoproteins, high degree of conservation of certain UGA codons, and demonstrated enzyme activity in the case of the HIV-1 GPx (32), strongly suggest that HIV-1 is able to do so. Potential RNA structures that may be involved in Sec insertion have been identified (including pseudoknot

associated with UGA codons), and preliminary evidence of Se-dependent readthrough suppression of the 3' UGA codon of *nef* is promising (33). More research on this question is obviously needed.

By our interpretation, the results of ^{75}Se labeling of HIV-infected cells reported by Gladyshev et al. (35), contrary to those authors' conclusions, are actually highly consistent with the possibility of in vitro expression of one or more of the predicted HIV-1 selenoproteins. In HIV-infected cells, Gladyshev et al. noted a distinct decline in levels of cellular selenoproteins—which is exactly what the HIV selenoprotein theory predicts (18,3l)-and an increase in "low molecular mass" Se- labeled material. Although they did not explicitly identify the actual mass range in question, it is evident from their data (35) (Fig. 1 *B)* that much of this novel ^{75}Se-labeled material lies immediately *above* the 6-kd band that they identify (page 836) as the lowest ^{75}Se-labeled band in uninfected cells. Hence, their conclusion that this result disproves the possibility of HIV-encoded selenoproteins seems premature considering that the predicted masses of the protease processed *pro-fs* and *env-fs* (vGPx) are in the range of 7 to 9 kd. ft is precisely this 9-kd form of *env-fs* that has been shown by Zhao et al. (32) to posses GPx enzyme activity.

Because NF-κB is a central factor in HIV-1 gene regulation and is also regulated by cellular selenoproteins and redox status, it is not really surprising that HIV-1 would have evolved to participate directly in those regulatory processes, by encoding its own selenoprotein modules. To understand fully interactions between HIV-1 and risk factors like malnutrition, Se status, and oxidative stress associated with drug abuse, this novel Se-based mechanism of HIV-1 gene regulation and pathogenesis will have to be fully elucidated.

EXPERIMENTAL SECTION

Sequence Analysis

Statistical significance of similarity between a query sequence (i.e., *env-fs* or *pro-fs)* and a prealigned protein family (GPx or NRD domain, respectively) was assessed using the BlockAlign program (Zhang, Kececioglu and Taylor, unpublished). This program produces an optimal, gapped alignment of a probe sequence against an exiting sequence alignment. By random shuffling of the query sequence, the average similarity score for optimally aligned random sequences of identical composition can be calculated. Significance is calculated a the distance of the actual core from the average random score in standard deviation. The blosum62 amino acid similarity matrix was used, with gap creation and gap extension penalties of 6, 2 for Figure 1 and 10, 2 for Figure 3. Sec residues were treated as Cys for the purpose of these calculations.

Molecular Modeling

The SYBYL program (Tripos Assoc., St. Louis, MO, U.S.A.) was used to build the putative HIV-1 vG Px homology model, starting from the bovine cellular GPx X-ray crystal structure, file J GP I from the Brookhaven Protein Database. The model was constructed based on the alignment of Figure 1, using the biopolymer protein loop option to reconnect regions where deletions were predicted from the alignment. The final model was minimized using the Kollman

united atom and all-atom force fields, with parameters for Sec estimated by analogy to Cys and from ab initio molecular orbital calculations on methyl selenol using the Gauian 94 program (Gaussian, Inc., Pittsburgh, PA, U.S.A.).

In Vitro -1 Frameshift Assay

A region of the HIV-1 *pol* gene, encompassing about 60 nucleotides spanning the potential frameshift site (31) (Fig. 4) was amplified with polymerase chain reaction from a laboratory strain of HIV-1, pBH-10 (vector obtained from the NIH AIDS Research and Reference Reagent Program, Ogden BioServices, Rockville, MD, U.S.A.), with incorporation of HindIII and Apal restriction sites in upstream and downstream oligonucleotide primers, respectively. The resulting protease sequence fragment was cloned into HindIII and Apal-digested pRW201 vector and on ligation with T4 DNA ligase, the recombinant plasmids (pro-fs-RW20 I) were transformed into *Escherichia coli* K-12 SU1675. The positive clones were confirmed and junctions were verified by sequencing the inserts in both directions. In vitro translation was performed by coupled reticulocyte lysate (TNT system, Promega, Madison, Wl, U.S.A.) with 1 µg of circular pro-fs-RW201 plasmid. Briefly, multiple 25 µl in vitro reactions were set up with and without RW20 I plasmid DNA added to 20 µl rabbit reticulocyte lysate mix, which includes T7 RNA polymerase and charged aminoacyl tRNAs. ^{35}S-Met label and water were added to a final volume of 25 µL and incubated at 30°C for 90 minutes. After adding the SDS-PAGE sample loading buffer the samples were denatured at 100°C for 3 minutes and then separated on 10% SDS-PAGE gels for 60 to 90 minutes at 90 V. The translation reaction products were electroblotted onto a neutrally charged nylon membrane for 35 minute at 23 V using the Semi-Phor System (Pharmacia-Upjohn, Kalamazoo, MI, U.S.A.). Finally, the membranes were exposed to X-OMAT Kodak x-ray film (Eastman-Kodak, Rochester, NY, U.S.A.) and developed. The film was then canned using the IS-1000 Digital Imaging System (Alpha Innotech Corp., San Leandro, CA, U.S.A.).

Acknowledgments: This work was supported by U.S. Public Health Service Grant RO1 DA11378 (National Institute on Drug Abuse, Rockville, Maryland, U.S.A.). We would also like to thank Marianna K. Baum, University of Miami School of Medicine, Miami, Florida, U.S.A., for her continued support, and John Atkin , University of Utah, Salt Lake City, Utah, U.S.A., for providing the RW20 I vector used in the -1 frameshift assay, as well as for the RW201-MMTV vector used as a positive control in Figure 4.

REFERENCES

1. Abrams B, Duncan D, Hertz-Picciotto I. A prospective study of dietary intake and acquired immune deficiency syndrome in HIV-seropositive homosexual men. *J Acquir Immune Defic Syndr* 1993;6:949-58.
2. Tang AM, Graham NM, Saah AJ. Effects of micronutrient intake on survival in human immunodeficiency virus type I infection. *Am J Epidemiol* 1996; 143: 1244-56.
3. Baum MK, Shor-Posner G. Lai S, et al. High risk of HIV-related mortality is associated with selenium deficiency. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997; 15:370-6.
4. Constans J, Pellegrin JL, Sergeant C, et al. Serum selenium predicts outcome in HIV infect ion. *J Acquir Immune Defic Syndr Hum Retrovirol* 1995;10:392.

5. Look MP, Rockstroh JK, Rao GS, et al. Serum selenium, plasma glutathione (GSH) and erythrocyte glutathione peroxidase (GSHPx)-levels in asymptomatic versus symptomatic human immunodeficiency virus-1 (HIV-1)-infection. *Eur J Clin Nutr* 1997:5 1:266-72.

6. Taylor EW, Bhat A, Nadimpalli RG, et al. HIV-1 encodes a sequence overlapping env gp41 with highly significant similarity to selenium-dependent glutathione peroxidases. *J Acquir Immune Defic Syndr Hum Retrovirol* 1997 ; 15:393-4.

7. McKenzie RC, Rafferty TS, Beckett GJ. Selenium: an essential element for immune function. *Immunol Today* 1998; 19:342-5.

8. Israel N, Gougerot-Pocidalo MA. Oxidative stress in human immunodeficiency virus infection. *Cell Mol Life Sci* 1997;53:864-70.

9. Allard JP, Aghdassi E, Chau J, et al. Oxidative stress and plasma antioxidant micronutrients in humans with HIV infection. *Am J Clin Nutr* 1998;67: 143-7.

10. Israel N, Gougerot-Pocidalo MA, Aillet F, et al. Redox status of cells influences constitutive or induced NF-KB translocation and HIV long terminal repeat activity in human T and monocytic cell lines. *J Immunol* 1992;149:3386-93.

11. Sappey, C, Legrand-Poels S, Best-Belpomme M, et al. Stimulation of glutathione peroxidase activity decreases HIV Type I activation after oxidative stress. *AIDS Res Hum Retroviruses* 1994; 10:1451-61.

12. Sunde RA. Selenium. In: O'Dell BL, Sunde RA, eds. *Handbook of Nutritionally Essential Minerals.* New York: Marcel Dekker, 1997:493-556.

13. Turner RJ, Finch JM. Selenium and the immune response. *Proc Nutr Soc* 1991;50:275-85.

14. Kiremidjian-Schumacher L, Roy M, Wishe HI, et al. Supplementation with selenium augments the functions of natural killer and lymphokine-activated killer cells. *Biol Trace Element Res* 1996; 52:227-39.

15. Roy M, Kiremidjian-Schumacher L, Wishe HI, et al. Selenium supplementation enhances the expression of interleukin 2 receptor subunits and internalization of interleukin 2. *Proc Soc Exp Biol Med* 1993;202:295-301.

16. Guimaraes MJ, Peterson D, Vicari A, et al. Identification of a novel seID homolog from eukaryotes, bacteria, and archaea: is there an autoregulatory mechanism in selenocysteine metabolism? *Proc Natl A cad Sci USA* l 996;93: 15086-91.

17. Clark LC, Combs GF Jr, Turnbull BW, et al. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin-a randomized controlled trial. *JAMA* l 996:276: 1957-63.

18. Taylor EW, Nadimpalli RG, Ramanathan CS. Genomic structures of viral agents in relation to the biosynthesis of selenoproteins. *Biol Trace Element Res* 1997;56:63-91.

19. Huggins ND, Khaled MA, Cornwell PE, et al. Nutritional status and immune function in cocaine and heroin abusers and in methadone treated subjects. *Res Commun Suhst Abuse* 1991;12:209-15.

20. Chandra R K. Nutrition and the immune system: an introduction. *Am J Clin Nutr* 1997;66:460S-3.

21. Seledtsov AM. Lipid peroxidation and status of the antioxidant system in opiate addiction, chronic alcoholism, or substance abuse caused by inhaling solvent vapors. *Vopr Med Khim* 1995;41:50-3.

22. Knight JA, Pieper RK, Smith SE, et al. Increased urinary lipoperoxides in drug abusers. *Ann Clin Lab Sci* 1988;18:374-7.

23. Devi BJ, Chan AW. Impairment of mitochondrial respiration and electron transport chain enzymes during cocaine-induced hepatic injury. *Life Sci* 1997;60:849-55.

24. Marmor M, Alcabes P, Titus S, et al. Low serum thiol levels predict shorter times-to-death among HIV-infected injecting drug users. *AIDS* 1997;11:1389-93.

25. Rodriguez Rodriguez EM, Sanz Alaejos M, Diaz Romero C. Urinary selenium concentrations in heroin abusers. *Clin Chim Acta* 1994;23 l :39-46.

26. Mitomo K, Nakayama K, Fujimoto K, et al. Two different cellular redox systems regulate the DNA-binding activity of the p50 subunit of NF-kappa Bin vitro. *Gene* 1994;145:197-203.

27. Kim IY, Stadtman TC. Inhibition of NF-kappaB DNA binding and nitric oxide induction in human T cells and lung adenocarcinoma cells by selenite treatment. *Proc Natl Acad Sci USA* 1997;94:12904-7.

28. Brigelius-Flohe R, Friedrichs B, Maurer S, et al. Interleukin-1-induced nuclear factor kappa B activation is inhibited by overexpression of phospholipid hydroperoxide glutathione peroxidase in a human endothelial cell line. *Biochem J* 1997;328: 199-203.

29. Hori K, Hatfield D, Maldarelli F, et al. Selenium supplementation suppresses tumor necrosis factor o.-induced human immunodeficiency virus type 1 replication in vitro *AIDS Res Hum Retroviruses* 1997;13:1325-32.

30. Gorlatov SN, Stadtman TC. Human thioredoxin reductase from HeLa cells: selective alkylation of selenocysteine in the protein inhibits enzyme activity and reduction with NADPH influences affinity to heparin. *Proc Natl Acad Sci USA* 1998;95:8520-5.

31. Taylor EW, Ramanathan CS, Jalluri RK, et al. A basis for new approaches to the chemotherapy of AIDS: novel genes in HIV-I potentially encode selenoproteins expressed by ribosomal frameshifting and termination suppression. *J Med Chem* 1994;37:2637-54.

32. Zhao L, Cox AG, Ruzicka J, et al. Molecular modeling and *in vitro* activity of an HIV-l-encoded glutathione peroxidase. *Proc Natl Acad* Sci *USA* 2000;97:6356-61.

33. Taylor EW, Nadimpalli RG, Dean RG, et al. Novel isoforms of HIV-1 *nef* are expressed by frameshifting and selenium-dependent suppression of UGA termination codons. *Antiviral Res* 1997;34:A23.

34. Aiken C, Trono D. Nef stimulates human immunodeficiency virus type 1 proviral DNA synthesis. *J Viral* 1995;69:5048-56.

35. Gladyshev VN, Stadtman TC, Hatfield DL, Jeang K-T. Levels of major selenoproteins in T cells decrease during HIV infection and low molecular mass selenium compounds increase. *Proc Natl Acad Sci USA* 1999;96:835-9.